THE PROTEIN COMPONENT OF HUMAN BRAIN THROMBOPLASTIN

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SUMMARY: The protein component of human brain tissue thromboplastin (factor III) has been purified by deoxycholate (DOC) extraction, ultracentrifugation, gel filtration and finally repeated preparative polyacrylamide gel electrophoresis (PGE) in the presence of sodium dodecylsulphate (SDS). The final preparations gave one band in analytical PGE. Reduced and alkylated protein appeared as a band of molecular weight about 53 000 in SDS-PGE.

The protein had a low solubility in aqueous solutions in the absence of detergents. When recombined with an optimal amount of the phospholipid fraction of tissue thromboplastin (fraction B) the procoagulant thromboplastin activity was regained. Neither alone nor after recombination with phospholipid did the protein catalyze the hydrolysis of aminoacyl-β-naphthylamides or casein.

INTRODUCTION: Tissue thromboplastin (factor III) is a protein - phospholipid complex which initiates blood coagulation in the extrinsic system by activating factor VII. By extraction of crude thromboplastin preparations with pyridine (1) or sodium deoxycholate (2) and subsequent gel filtration (3) a protein fraction (factor III apoprotein) and a phospholipid fraction (fraction B) can be separated. Alone each fraction is inactive, when recombined they regain full procoagulant activity.

Nemerson and Pitlick (4) described the purification of a protein fraction from bovine lung which had high procoagulant activity when recombined with phospholipids and which also had peptidase activity (5), and they suggested that this activity was important for the activation of factor VII. In contrast, our earlier results (6) indicated that the activation of factor VII by tissue thromboplastin is reversible and therefore probably does not involve peptide bond cleavage. Further evidence in

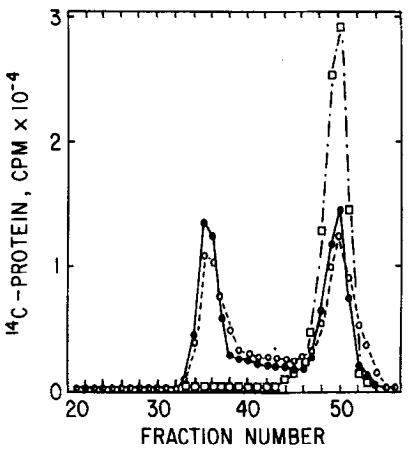

Figure 1. Gel filtration in SDS of samples containing [^{14}C]protocollagen and [^{14}C]procollagen after limited proteolytic digestion. Tendon cells (7), 7.8 x 10^8, were incubated under N_2 in 40 ml of modified Krebs medium containing 100 µC of [^{14}C]proline. The medium was deaerated and equilibrated with N_2 beforehand, and the incubation was performed in an Erlenmeyer flask continously flushed with N_2. After 30 min, 4.1 mg of cycloheximide in 1 ml of medium was added and 10 min later the system was exposed to O_2. Aliquots of 3 ml were removed at 2, 5 and 60 min after exposure to O_2 and they were rapidly pipetted into 0.5 ml of 5.5 N acetic acid containing 350 µg of pepsin and 0.25 M iodoacetamide (Fisher Scientific) at 15°. The sample was immediately homogenized at 15°, and incubated at 15° for 6 hours. The pepsin digest was then dialyzed at 4° against 0.4 M NaCl and 0.1 M Tris-HCl, pH 7.4, overnight. After adjustment to 2% SDS and 0.1 M sodium phosphate, pH 7.4, by adding a one-tenth volume of a concentrated stock solution, the sample was incubated at 100° for 3 min and at 37° for 3 hours prior to gel filtration on 6% agarose in 0.1% SDS and 0.1 M sodium phosphate (9). The V_o of the column was 54 ml (fraction 27) and the V_t was 136 ml (fraction 68). α-Chains of acid soluble calf skin collagen eluted in fractions 35-36. Symbols: Sample removed at the end of incubation under N_2 (□··□); sample removed 5 min after exposure to O_2 (0---0); sample removed 60 min after exposure to O_2 (●—●).

rate for helix formation by either procollagen or protocollagen polypeptides has not been measured.

Materials and Methods. Cells were prepared by enzymic digestion of leg tendons from 17-day old chick embryos and were incubated in modified Krebs medium. Unless otherwise indicated, experimental conditions and the source of materials were the same as previously specified (7).

Rate of Helix Formation at 37° after Intracellular Hydroxylation of Protocollagen to Procollagen. Tendon cells were allowed to synthesize and accumulate [^{14}C]protocollagen by incubating them with [^{14}C]proline under

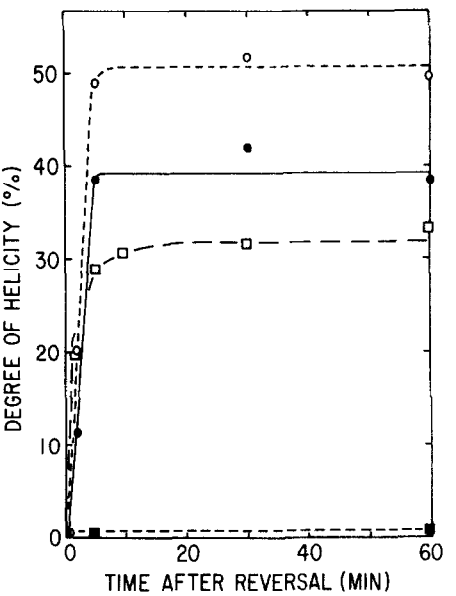

Figure 2. Rate of helix formation with hydroxylation of intracellular protocollagen to procollagen at 37° or with cooling of intracellular protocollagen. In experiment I the rate of helix formation was followed after exposure of the intracellular protocollagen to 0_2 as in Fig. 1. The degree of helicity was estimated as the fraction of ^{14}C-protein or of [^{14}C]hydroxyproline (5) in the chromatogram which eluted in the same position as α chains after pepsin digestion. In experiment II, 6.3 x 10^6 tendon cells were incubated at 37° in 85 ml of modified Krebs medium containing 0.3 mM α,α´-dipyridyl and 15 μC of [^{14}C]proline. After 90 min, 8.6 mg of cycloheximide in 1 ml of medium was added. After 10 min, half the sample was adjusted to 20 mM DTT and the incubation was continued an additional 40 min. The samples were cooled to 15° and the degree of helicity tested as in Fig. 1. Symbols: Helicity as measured by ^{14}C-protein in experiment I (●——●);helicity as measured by [^{14}C]hydroxyproline in experiment I (0---0); helicity as measured by ^{14}C-protein in control cells in experiment II (□- -□); helicity as measured by ^{14}C-protein in cells treated with DTT in experiment II (■- - -■).

anaerobic conditions (6) (Fig. 1). Further protein synthesis was stopped by adding cycloheximide and the cells were exposed to atmospheric 0_2 so that the accumulated [^{14}C]protocollagen was hydroxylated to [^{14}C]procollagen. The samples were then subjected to pepsin digestion and the ^{14}C-protein recovered as α chains after the digestion was used as an estimate of the pro-α chains of [^{14}C]protocollagen or [^{14}C]procollagen which were in a triple-helical conformation (Fig. 1). (For data substantiating the validity of this test see references 5,8-12). In cells not exposed to 0_2, none of the pro-α chains resisted pepsin digestion at 15° if the procedure previously employed (5) was altered so that the digestion was initiated as soon as the samples were cooled to 15°. However,

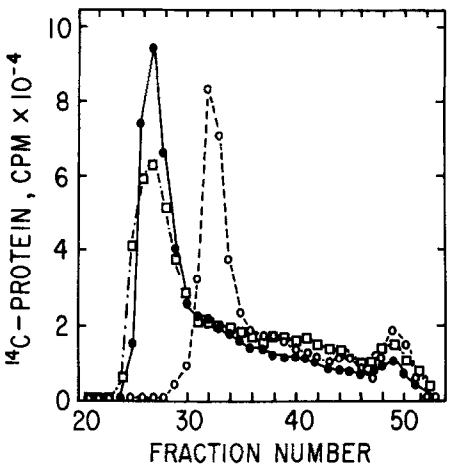

Figure 3. Reversible reduction of intracellular protocollagen with DTT. Tendon cells, 2 x 10^8, were incubated in 25 ml of modified Krebs medium containing 10% fetal calf serum, 0.3 mM α,α′-dipyridyl and 50 μC of [^{14}C]proline. After 60 min, 2.6 mg of cycloheximide in 1 ml of medium was added and the incubation was continued for 10 min. A 3-ml aliquot was then removed and the rest of the sample was incubated with 20 mM DTT for 40 min. Another 3-ml aliquot was removed and the remainder of the sample was centrifuged at 1200 x g to separate cells from medium. The cells were washed five times with 10 ml of modified Krebs medium containing 10% fetal calf serum, 0.3 mM α,α′-dipyridyl and 100 μg cycloheximide per ml. The cells were finally resuspended in 10 ml of the medium and incubated another 60 min. In each case the samples were rapidly pipetted into 1 ml of 10% SDS, 0.5 M iodoacetamide, and 0.1 M sodium phosphate, pH 7.4. They were immediately heated to 100° for 3 min to inactivate endogenous proteases and incubated a further 3 hours at 37° prior to gel filtration as in Fig. 1. Symbols: Non-dialyzable ^{14}C-protein in the sample removed before DTT treatment (●—●); non-dialyzable ^{14}C-protein in the sample removed at the end of incubation with DTT (0---0); non-dialyzable ^{14}C-protein in the sample after the cells were washed and incubated in the medium not containing DTT (□·—·□).

the amount of ^{14}C-protein which resisted digestion rapidly increased after the cells were exposed to 0_2, and it reached a maximal value in about 5 min (Fig. 2). The apparent rate of helix formation was the same whether the process was followed by total ^{14}C-protein recovered as α chains after the enzymic digestion or by [^{14}C]hydroxyproline recovered as α chains under the same conditions.

Rate of Helix Formation on Cooling of Intracellular Protocollagen. The rate of helix formation was also examined by allowing the cells to accumulate non-helical [^{14}C]protocollagen at 37° and then cooling the cells to 15°, a temperature which is below the T_m of the molecule (6,12-14). The degree of helicity of the [^{14}C]protocollagen reached a maximal value after the cells had

Table I. Degree of helicity after treatment of intracellular protocollagen with DTT. Experimental conditions were as in Fig. 3. Values were expressed as cpm of ^{14}C-protein recovered as α chains after proteolytic digestion of a sample containing 10^5 cpm of non-dialyzable ^{14}C-protein as in Fig. 1.

TREATMENT	^{14}C-PROTEIN RESISTANT TO PROTEOLYTIC DIGESTION (cpm x 10^{-3})
None	18.7
DTT	3.9
DTT followed by incubation without DTT	12.3

been cooled to 15° for about 5 min (Fig. 2). As reported elsewhere, the polypeptides recovered as α chains after pepsin digestion under these conditions were shown to be derived from protocollagen by their ability to serve as substrates for prolyl hydroxylase (5,12), and their amino acid composition (12).

Role of Disulfide Bonds in Facilitating Helix Formation. The role of disulfide bonds in formation of the triple-helix was examined by treating tendon cells with DTT, a technique recently used to produce dissociation of prolyl hydroxylase in fibroblasts (15). The cells were allowed to accumulate [^{14}C]protocollagen by incubation in the presence of the iron chelator α,α´-dipyridyl (5), further protein synthesis was stopped by adding cycloheximide, and then the cells were treated with 20 mM DTT. In cells not treated with DTT most of the [^{14}C]protocollagen eluted from the SDS-agarose in a peak with an apparent molecular weight larger than pro-α chains if the protein was not reduced with mercaptoethanol prior to chromatography (Fig. 3), but it eluted as pro-α chains if it was reduced (see reference 5). The results indicated therefore that the pro-α chains in the protocollagen were linked by interchain disulfide bonds. In contrast, most of the ^{14}C-protein from cells treated with DTT was recovered as pro-α chains when the cell homogenates were chromatographed without further reduction. When the cells treated with DTT were cooled to 15° for 60 min, essentially all the ^{14}C-protein remained digestible with pepsin

(Fig. 2). It was apparent therefore that the DTT had penetrated the cells so as to reduce the interchain bonds among the pro-α chains of the protocollagen, and that these events were accompanied by a failure of the intracellular protocollagen to become helical on cooling.

Further experiments demonstrated that reduction of the intracellular protocollagen with DTT was a reversible process. Cells were incubated with DTT and then the reagent was removed by repeated washings of the cells. Under these conditions, a large fraction of the intracellular ^{14}C-protein re-assembled into a form larger than pro-α chains (Fig. 3). Also, after removal of the DTT, the intracellular pro-α chains became helical on cooling the cells to 15° (Table I).

Discussion. It has not been conclusively established that the procollagen from all tissues contains interchain disulfide bonds (4), but the most complete form of the molecule from the tendon cells (4,16) and apparently from several other sources (4) has been shown to consist of three pro-α chains linked by disulfide bonds. In the case of tendon procollagen it has been demonstrated that these interchain bonds are formed among the NH_2-terminal extensions of the three pro-α chains, and that the interchain bonds form at about the same time as the newly-synthesized pro-α chains become triple-helical (16).

Although procollagen has been isolated in sufficient quantities for limited chemical studies, it has not previously been possible to study its renaturation because the native protein is difficult to purify as an intact molecule (4) and is frequently associated with proteases which hydrolyze the protein when it is denatured (Uitto, J., Berg, R.A., and Prockop, D.J., unpublished observations). In the present experiments, therefore, cells were allowed to accumulate [^{14}C]-protocollagen which has a T_m of about 24° and is non-helical during its synthesis in cells at 37° (6,12-14). The rate of helix formation was then examined either as the accumulated [^{14}C]protocollagen was hydroxylated to [^{14}C]procollagen in cells, or the cells were cooled below the T_m of protocollagen. In both instances, the

protein became triple-helical within about 5 min, or in a fraction of the time required for isolated α chains to become helical (2,3). When disulfide bonds in the NH_2-terminal extensions of protocollagen (4,16) were reduced by treating the cells with DTT, the rate of helix formation was markedly decreased. The results demonstrated therefore that the NH_2-terminal extensions found in protocollagen and procollagen, but not in collagen, greatly accelerate the rate at which the triple-helix is formed.

Treatment of the cells with DTT was shown to reduce interchain bonds but it may also have reduced intrachain disulfides (4,17) which are necessary to maintain conformations of the individual NH_2-terminal extensions; these conformations may in themselves promote the required chain association. However, reduction of intact proteins under non-denaturing conditions does not generally destroy structure (18,19). It is therefore possible that DTT did not change the conformation of the NH_2-terminal extensions. If this is true, it would follow that the correct conformations of the NH_2-terminal extensions are not in themselves sufficient to produce chain association and that pro-α chains must be linked by interchain bonds before the triple-helix is assembled during the biosynthesis of procollagen.

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